TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. §371

Attorney Docket Number: 9325-0007.10

U.S. Application No. (if known, see 37 CFR §1.5): 09/555674

International Application No.	International Filing Date	Priority Date Claimed
PCT/IL98/00586	December 1, 1998	December 4, 1997

Title of Invention: COMBINED CHEMO-IMMUNOTHERAPY WITH LIPOSOMAL DRUGS AND CYTOKINES

Applicant(s) for DO/EO/US: Alberto A. Gabizon, Eliezer Kedar, and Yechezkel Barenholz

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ **This is a FIRST submission of items concerning a filing under 35 U.S.C. §371.**
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. §371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. §371(b) and PCT Articles 22 and 39(1).
4. ☒ **A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.**
5. ☒ **A copy of the International Application as filed (35 U.S.C. §371(c)(2))**
   - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ☒ **has been transmitted by the International Bureau.**
   - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. §371(c)(2)).
7. ☒ **Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))**
   - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ☐ have been transmitted by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   - d. ☒ **have not been made and will not be made.**
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ **An oath or declaration of the inventor(s) (35 U.S.C. §371(c)(4)) (unsigned).**
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR §1.97 and §1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR §3.28 and §3.31 is included.
13. ☐ **A FIRST preliminary amendment.**
    ☐ A SECOND or SUBSTITUTE preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of Power of Attorney and/or Address letter.
16. ☒ **Other items or information: Certificate of Express Mail**

U.S. Application No. (if known, see 37 CFR 1.5)	International Application No.	Attorney's Docket No.
09/555674	PCT/IL98/00586	9325-0007.10

17. ☒ **The following fees are submitted:**

	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR $1.492(a)(1)-(5):		
Search Report has been prepared by the EPO or JPO ..... $ 840.00		
International preliminary examination fee paid to USPTO (37 CFR $1.482) ..... $ 670.00		
No international preliminary examination fee paid to USPTO (37 CFR $1.482) but international search fee paid to USPTO (37 CFR $1.445(a)(2)) ..... $ 760.00		
Neither international preliminary examination fee (37 CFR $1.482) nor international search fee (37 CFR $1.445(a)(2)) paid to USPTO. $970.00		
International preliminary examination fee paid to USPTO (37 CFR $1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) ..... $ 96.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$130.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claim priority date (37 CFR $1.492(e)).	$130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	30 - 20 =	10	10 x $ 18.00	$180.00	
Independent Claims	3 - 3 =	0	0 x $ 78.00	$ 00.00	
Multiple Dependent Claim(s) (if applicable)			+ $260.00	$ 00.00	
			TOTAL OF ABOVE CALCULATIONS =	$1,150.00	
Reduction of ½ for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR $1.9, $1.27, $1.28)				$	
			SUBTOTAL =	$	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR $1.942(f)).			+	$	
Fee for recording the enclosed assignment (37 CFR $1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR $3.28, $3.31). $40.00 per property.			+	$	
			TOTAL FEES ENCLOSED =	$1,150.00	
				Amount to be refunded	$
				charged	$

a. ☒ **A check in the amount of $1,150.00 to cover the filing fees is enclosed.**

b. ☐ Please charge Deposit Account No. 04-0531 in the amount of $* to cover the above fees. This sheet is provided in triplicate.

c. ☒ **The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 04-0531. This sheet is provided in triplicate.**

NOTE: Where an appropriate time limit under 37 CFR $1.494 or $1.495 has not been met, a petition to revive (37 CFR $1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

June 2, 2000
DATE

LeeAnn Gorthey
Signature

Iota Pi Law Group
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Registration No.: 37,337

Attorney Docket No.: 9325-0007.10

Applicant: **Alberto A. Gabizon et al.**

Serial No.: 09/555,674

Filing Date: **June 2, 2000**

For: COMBINED CHEMO-IMMUNOTHERAPY WITH LIPOSOMAL DRUGS AND CYTOKINES

Small Entity Statement Under 37 CFR 1.9(f) and 1.27(c) - Small Business Concern

I hereby declare that I am: *

☐ The owner of the small business concern identified below:

☒ **An official of the small business concern empowered to act on behalf of the concern identified below:**

Name of Concern: **Hadasit Medical Research Services and Development Ltd.**

Address of Concern: **Kiryat Hadassah, P.O.B. 12000, Jerusalem 91120, Israel**

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR §121.12, and reproduced in 37 CFR §1.9(d), for purposes of paying reduced fees under 35 U.S.C. §41 in that the number of employees, including those of its affiliates, does not exceed 500 persons and the concern has not assigned, granted, conveyed, or licensed, and is under no obligation under contract or law to assign, grant, convey, or licenses, any rights in the invention to any person who could not be classified as an independent inventor if that person had made the invention, or to any concern which would not qualify as a small business concern or a nonprofit organization under this section. For this section, concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both. The number of employees of the business concern is the average over the fiscal year of the persons employed during each of the pay periods of the fiscal year. Employees are those persons employed on a full-time, part-time or temporary basis during the previous fiscal year of the concern.

I hereby declare that rights under contract or law in the above-identified application have been conveyed to and remain with the small business concern identified above.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR §1.9(d) or by any concern which would not qualify as an independent inventor under 37 CFR §1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR §1.9(d) or a nonprofit organization under 37 CFR §1.9(e).

NOTE: Separate statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR §1.27).

Attorney Docket No.: 9325-0007.10

Name: Yissum Research Development Company of the Hebrew University of Jerusalem

Address: Jabotinsky Street 46, Jerusalem 91042, Israel

☐ individual ☒ **small business concern** ☐ nonprofit organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate (37 CFR §1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this statement is directed.

NAME OF PERSON SIGNING: RAPHAEL HOFSTEIN PH.D. MANAGING DIRECTOR HADASIT MEDICAL RESEARCH SERVICE & DEVELOPMENT LTD.

TITLE OF PERSON SIGNING:

ADDRESS OF PERSON SIGNING:

הדסית
שרותי מחקר רפואי ופיתוח
hadasit
medical research services
& development ltd

Signature: Date: 5/6/2000

Attorney Docket No.: 9325-0007.10

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Serial No.: **09/555,674**

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Attorney Docket No.: 9325-0007.10

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this statement is directed.

NAME OF PERSON SIGNING: Mordehai Perlmutter

TTTLE OF PERSON SIGNING: Managing Director & CEO

ADDRESS OF PERSON SIGNING: 46 Jabotinsky St., POB 4279, Jerusalem 91042, ISRAEL

Signature: ______________________ Date: JUNE 19, 2000

COMBINED CHEMO-IMMUNOTHERAPY WITH LIPOSOMAL DRUGS AND CYTOKINES

Field of the Invention

The present invention relates to a method and composition for antitumor therapy, and more particularly to combination therapy using a chemotherapeutic drug and an immunostimulating cytokine. Sequential administration of these two components, both encapsulated in liposomes, is shown to have a significant antitumor effect as compared to administration of the individual components, in free form or in liposomes.

References

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Woodle, M.C. *et al.*, U.S. Pat. No. 5,013,556 (1991).

Background of the Invention

Despite prolific research in the area of cancer chemotherapy, such treatment remains far from satisfactory. The inability of chemotherapeutic drugs to reach the tumor site, intrinsic and acquired cross-resistance to multiple chemotherapeutic agents, and, especially, the high toxicity of many of these agents all contribute to treatment failures.

The use of immunostimulating cytokines, such as IL-2 and interferon-α, has proven to be effective in treatment of a proportion of patients with malignancies such as melanoma and renal cell carcinoma, both alone and in combination with other therapeutic agents. However, major problems limit their wide clinical use, including rapid plasma clearance, biodistribution to nonrelevant tissues, and high toxicity. Furthermore, their efficacy has been low in treatment of the most common tumors, *e.g.* colorectal, mammary, prostate, and lung carcinomas.

Summary of the Invention

The present invention includes, in one aspect, a method of antitumor therapy, which comprises administering to a subject in need of such treatment, a therapeutically effective amount of a combination of a chemotherapeutic drug and an immunostimulating cytokine, both encapsulated in liposomes. In another aspect, the invention provides a composition for use in antitumor therapy, which comprises such a combination of a chemotherapeutic drug and an immunostimulating cytokine, both encapsulated in liposomes. Administration of the combination produces a greater therapeutic effect than a combination of the effects produced by the liposome-encapsulated components administered individually.

The invention also includes a method of antitumor therapy in which a chemotherapeutic drug, encapsulated in liposomes, is administered in combination with a cytokine, which may or may not be encapsulated in liposomes. In this method, the drug is encapsulated in liposomes which contain about 1-10 mole percent of a lipid having a polar head group derivatized with a polyethylene glycol (PEG) chain which has a molecular weight of between 750 and 10,000 daltons. The therapeutic effect of this combination is greater than a combination of the effects produced by the liposome-encapsulated drug and the cytokine administered individually.

In all cases, administration of the cytokine preferably follows administration of the liposome-encapsulated drug.

The chemotherapeutic drug is preferably selected from cis-platin, a chemotherapeutic anthraquinone, and a topoisomerase I inhibitor, such as camptothecin or a camptothecin analog. More preferably, the drug is adriamycin (doxorubicin), in which case the liposome-encapsulated form of the drug is preferably DOXIL®.

The immunostimulating cytokine is preferably selected from the group consisting of interleukin-2 (IL-2), IL-12, IL-15, IL-18, IFN-γ, IFN-α, IFN-β, TNF-α, G-CSF, and GM-CSF. More preferably, the cytokine is IL-2.

The encapsulating liposomes employed in the composition and method preferably contain at least one lipid selected from dimyristoyl phosphatidyl choline (DMPC), dimyristoyl phosphatidyl glycerol (DMPG), 1,2-distearoyl-3-trimethylammonium propane (DSTAP), phosphatidyl choline, phosphatidyl ethanolamine, and cholesterol.

The liposomes may be small unilamellar vesicles (SUV), defined as having a mean diameter of approximately 20 to 100 nm, or large unilamellar vesicles (LUV), defined as having a mean diameter of approximately 100 to 200 nm. Such liposomes preferably contain about 1-10 mole percent of a lipid having a polar head group derivatized with a polyethylene glycol (PEG) chain which has a molecular weight of between 750 and 10,000 daltons.

Alternatively, the liposomes may be large multilamellar vesicles (MLV) having a mean

diameter of approximately 250 to 2000 nm. The MLV may also contain a PEG-derivatized lipid as described above.

In preferred embodiments, the chemotherapeutic drug is encapsulated in vesicles having a mean diameter of approximately 50 to 120 nm, and containing about 1-10 mole percent of a lipid having a polar head group derivatized with a polyethylene glycol (PEG) chain as described above. In another preferred embodiment, the cytokine is encapsulated in liposomes containing dimyristoyl phosphatidyl choline (DMPC) plus 0 to 50 mole percent of at least one lipid selected from dimyristoyl phosphatidyl glycerol (DMPG) and 1,2-distearoyl-3-trimethylammonium propane (DSTAP).

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figure 1 shows the survival rate of BALB/c mice injected intraperitoneally with 5×10^5 M109 tumor cells (lung adenocarcinoma) and subsequently treated with free adriamycin or DOXIL®, respectively, alone or in combination with intraperitoneal IL-2 in DMPC/DMPG MLV liposomes, or with liposomal IL-2 alone; and

Figure 2 shows the survival rate of BALB/c mice injected intravenously with 5×10^5 M109 tumor cells and subsequently treated with DOXIL® (at day 7), alone or in combination with intravenous IL-2 in Stealth® PEGylated SUV liposomes (at days 11, 14, and 17), or with liposomal IL-2 alone (at days 11, 14, and 17).

Detailed Description of the Invention

I. Definitions

The terms below have the following meanings unless indicated otherwise.

"Vesicle-forming lipids" refers to amphipathic lipids which have hydrophobic and polar head group moieties, and which (a) can form bilayer vesicles in water, as exemplified by phospholipids, or (b) can be stably incorporated into lipid bilayers, with the hydrophobic moiety in contact with the interior, hydrophobic region of the bilayer membrane, and the polar head group moiety oriented toward the exterior, polar surface of the membrane.

The vesicle-forming lipids of this type typically include one or two hydrophobic acyl hydrocarbon chains or a steroid group, and may contain a chemically reactive group, such as an amine, acid, ester, aldehyde or alcohol, at the polar head group. Included in this class are the phospholipids, where the two hydrocarbon chains are typically between about 14-22 carbon atoms

in length, and have varying degrees of unsaturation. Representative examples are phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidic acid (PA), phosphatidyl inositol (PI), sphingomyelin (SM), negatively charged lipids such as dimyristoyl phosphatidyl glycerol (DMPG), and positively charged lipids such as 1,2-distearoyl-3-trimethylammonium propane (DSTAP). The liposomes may also contain sterols, such as cholesterol, which do not form liposomes themselves but can be incorporated into, and may stabilize, liposomes containing lipids such as those described above.

A "Cetus unit" (CU) is equal to six International Units (IU) of Immunological Activity, the international reference standard of a biological preparation of interleukin-2 (IL-2). The term "unit" used herein in reference to cytokine levels refers to Cetus units.

II. Liposomal Compositions

A. Lipid Components

Various vesicle-forming lipids, as defined above, may be used in the present liposomal compositions, according to methods well known in the art. Preferred lipids for the current invention allow long-term storage of the liposome-entrapped agents and effective release of these components upon administration. Representative lipids include, but are not limited to, dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), cholesterol, egg phosphatidylcholine (egg PC), phosphatidyl ethanolamine (PE), distearoyl phosphatidyl ethanolamine (DSPE), phosphatidyl inositol (PI), 1,2-distearoyl-3-trimethylammonium propane (DSTAP), 1,2-dimyristoyl-3-trimethylammonium propane (DMTAP), and combinations thereof.

The vesicle-forming lipids, preferably those making up SUV's, may contain about 1-10 mole percent of a lipid having a polar head group, typically a phosphate containing head group, derivatized with a polyethylene glycol (PEG) chain which has a molecular weight of between 750 and 10,000 daltons. The rate of clearance of liposomes from circulation is typically reduced by employing such PEG-derivatized, or "PEGylated", lipids. PEG coating is believed to inhibit nonspecific adsorption of serum proteins, thereby preventing nonspecific recognition of liposomes by macrophages (Papahadjopoulos, *et al.*, 1991). Another advantage of these long-circulating liposomes is their good extravasation capacity and high accumulation in tumors (Lasic and Martin, 1995; Gabizon, *et al.*, 1997). They are also referred to as sterically stabilized liposomes, SSL, or Stealth® liposomes.

The preparation of such lipids is described in, for example, Woodle, *et al.*, 1991; Sears (1984, 1985); Tirosh *et al.* (1997) or copending and co-owned application having U.S. serial number 08/570,440. The PEG chain may be linked directly to the phosphatidic acid head group of a phospholipid. Various other linkages are possible; for example, lipids containing a phosphatidyl

ethanolamine (PE) or other amino head group may be conveniently coupled to activated PEG chains via reaction with brominated PEG. PEG-modified lipids are also commercially available, *e.g.* from Sequus Corporation, Menlo Park, CA.

B. Preparation of Liposomes and Liposomal Compositions

Liposomes may be prepared by a variety of techniques, such as those detailed in Szoka *et al.* (1980b). To form multilamellar vesicles (MLV's), a mixture of vesicle- forming lipids dissolved in a suitable solvent is evaporated in a vessel to form a thin film, which is then hydrated by an aqueous medium to form MLV's, typically with sizes between about 0.1 to 10 microns. Tert-butanol is a preferred solvent for the process. The MLV's may then be downsized to a desired size range by extruding the aqueous suspension through a polycarbonate membrane having a selected uniform pore size, typically 0.05 to 1.0 microns.

Preparations of MLV's or REV's (described below) may be treated, *e.g.* by extrusion, sonication or high pressure homogenization, to produce unilamellar vesicles. Small unilamellar vesicles (SUV's) are characterized by sizes in the 30-100 nm range, while large unilamellar vesicles (LUV's) are defined as those having mean diameters of about 100-200 nm. SUV's may also be formed directly by high pressure homogenization of an aqueous dispersion of lipids.

Various methods are available for encapsulating other agents in liposomes. Preparation of SSL-encapsulated IL-2 is described in Kedar *et al.* (1994). In this procedure, generally, the lipid components, including a PEG-substituted lipid, are dissolved in t-butanol. The solution is sonicated, and IL-2 is added with further sonication. The mixture is lyophilized and rehydrated, forming MLV's, which can then be downsized by high pressure homogenization or by successive extrusion through polycarbonate filters. These downsizing methods gave vesicles having diameters of 50-80nm and about 200nm, respectively. The procedure achieved approximately 80-90% encapsulation of the IL-2.

In the reverse phase evaporation method (Szoka, *et al.*, 1980a) a nonaqueous solution of vesicle-forming lipids is dispersed with a smaller volume of an aqueous medium to form a water–in-oil emulsion. The agent to be incorporated is included either in the lipid solution, in the case of a lipophilic agent, or in the aqueous medium, in the case of a water-soluble agent. After removal of the lipid solvent, the resulting gel is converted to liposomes. These reverse phase evaporation vesicles (REVs) have typical average sizes between about 0.2-4 microns and are predominantly oligolamellar, that is, containing one or a few lipid bilayer shells. The REVs may be sized by extrusion, if desired, to give oligolamellar vesicles having a maximum selected size between about 0.05 to 1.5 microns.

Other methods for adding additional components to liposomal compositions include colyo-philization with other components and redispersion of the resulting solid to form MLV's. In a

method described by Adler, *et al.* (1995), an aqueous solution of the agent to be encapsulated is added to a t-butanol solution of lipids. The mixture is sonicated and lyophilized, and the resulting powder is rehydrated.

Liposome compositions containing an entrapped agent may be treated after final sizing, if necessary, to remove free (non-entrapped) agent. Conventional separation techniques, such as centrifugation, diafiltration, and molecular-sieve chromatography are suitable for this purpose. The composition may also be sterilized by filtration through a conventional 0.22 or 0.45 micron depth filter.

To form the compositions of the current invention, the concentration of drug and/or cytokine in the liposomes is preferably effective to give a protein/lipid weight ratio between about 1:100 and 1:1000.

Stabilizers may also be added to the liposomal compositions. For example, addition of a metal chelator such as Desferal™ or diethylenetriamine pentaacetic acid (DTPA) to the lyophilization medium, at a concentration of 100 μM, has been shown to reduce activity loss of entrapped IL-2 during liposome preparation and storage at 4°C. Antioxidants such as BHT or Vitamin E may also be included.

For long term storage, the compositions may be stored as the dry lyophilized powder, which is stable for at least a year at 4°C, and hydrated to form an aqueous suspension before use.

III. Combined Chemotherapy/Cytokine Therapy

A. Formulations

Cytokines useful for enhancing antitumor activity of chemotherapeutic drugs include IL-2, IL-12, IL-15, IL-18, IFN-γ, IFN-α, IFN-β, TNF-α, G-CSF, and GM-CSF. A preferred cytokine for the present invention is IL-2 (interleukin 2), which acts as a growth and maturation factor for T-lymphocytes.

A variety of liposomal formulations may be used for encapsulation of the cytokine. These include MLV, LUV or SUV, as defined above, as well as OLV (oligolamellar vesicles) and MVV (multivesicular vesicles), composed of vesicle-forming lipids such as those described above. Combinations of lipids are generally most effective (see, for example, Kedar *et al.*, 1994). One preferred type of formulation employs SUV or LUV, having a mean diameter of approximately 50 to 120 nm, containing about 1-10 mole percent of a lipid having a polar head group derivatized with a polyethylene glycol (PEG) chain (also referred to as a PEGylated lipid). Formulation A below is one example. Other preferred formulations employ dimyristoyl phosphatidyl choline (DMPC) and, optionally, up to 50 mole percent of at least one lipid selected from dimyristoyl phosphatidyl glycerol (DMPG) and 1,2-distearoyl-3-trimethylammonium propane (DSTAP). In

these formulations, the proportion of DMPG and/or DSTAP is more preferably 5 - 25 mole percent. Formulation B below is one example. In all cases, small quantities (up to about one mole percent) of stabilizers such as tocopherol or Desferal™ may be included.

For the experiments described below, liposomal IL-2 was prepared in two formulations, using IL-2 obtained from Chiron Corporation (Emeryville, CA), according to known methods such as those described above. Formulation A employed sterically stabilized (SSL) small unilamellar vesicles (SUV) composed of ^{2000}PEG-DSPE (N-carbamyl-(polyethylene glycol methyl ether)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt, provided by Sequus Corporation), egg phosphatidyl choline, and cholesterol in a molar ratio of about 5:55:40. The vesicles were about 50-70 nm in diameter. Encapsulation efficiency of IL-2 was greater than 80%, based on an *in vitro* IL-2 bioassay (*i.e.*, > 80% of the initial amount of added IL-2 became encapsulated in liposomes).

Formulation B employed multilamellar vesicles (MLV) composed of DMPC-DMPG (dimyristoyl phosphatidyl choline - dimyristoyl phosphatidyl glycerol) in a 9:1 molar ratio. The vesicles were approximately 500-1500 nm in size, and the encapsulation efficiency was approximately > 90%. This high efficiency of encapsulation was achieved at a lipid:IL-2 ratio (wt:wt) of 1000:1 for DMPC alone, and 100:1 for DMPC containing DMPG or DSTAP.

The chemotherapeutic drug is preferably encapsulated in liposomes having about 1-10 mole percent of a PEGylated lipid, as described above. For example, DOXIL®, a stable formulation of adriamycin in Stealth® liposomes, is available from SEQUUS Pharmaceuticals, Inc. (Menlo Park, CA). Free adriamycin is available, *e.g.*, from Cetus Oncology Corp. (Emeryville, CA) as a formulation of doxorubicin hydrochloride and lactose.

Other chemotherapeutic drugs which are also preferred for the present method include other anthraquinones, such as epirubicin, daunorubicin, and mitoxanthrone, and cis-platin. Also contemplated are topoisomerase I inhibitors such as camptothecin and its analogs, *e.g.* topotecan and irinotecan, also designated CPT-11. Camptothecin is isolated from the stem wood of the Chinese tree *Camptotheca aciminata*; preparation of the above noted analogs has been described by, *e.g.*, Curran *et al.* (1996).

B. Liposomal Adriamycin - Liposomal IL-2

The effect of adriamycin, used alone or in combination with interleukin-2 (IL-2), where each component was in free or liposome-encapsulated form, on the survival rate of BALB/c mice infected with tumor cells, was tested as described below.

B1. Lung Adenocarcinoma Model: IL-2 in MLV. Six groups of BALB/c mice were injected intraperitoneally with 5×10^5 M109 tumor cells (day 0). Free adriamycin or DOXIL®, respectively, were administered intravenously on day 7 at a dose of 8 mg/kg, and intraperitoneal

cytokine treatment was initiated 3 days later. Liposomal IL-2 (formulation B; MLV DMPC/DMPG (9:1 mole ratio) liposomes containing IL-2) was given once daily (50,000 CU/mouse) on days 10, 13 and 16. Control groups received no treatment or received the IL-2 treatment alone.

Each group, consisting of 8-9 mice, was inspected for survival up to 100 days after tumor inoculation. Table I shows the number of survivors at the end of the experiment and the median survival time obtained; Figure 1 shows the survival curves for all groups.

TABLE I

GROUP	TREATMENT	NUMBER OF SURVIVING MICE/TOTAL	MEDIAN SURVIVAL (DAYS)
1	Control	2/8	54
2	ADR	0/8	42
3	ADR + MLV-IL-2	5/8	> 100
4	DOXIL®	5/8	> 100
5	DOXIL® + MLV-IL-2	8/8	> 100
6	MLV-IL-2	1/9	21

As Table I shows, adriamycin (ADR) in combination with MLV-IL-2 (liposomal IL-2, formulation B) was much more effective than either adriamycin alone or liposomal IL-2 alone, both of which showed lower survival rates than the control. When liposomal adriamycin (DOXIL®) was administered alone, or when non-liposomal adriamycin was combined with liposomal IL-2, five of eight mice survived for the duration of the test.

The best result, *i.e.* survival of all subjects for 100 days or more, was observed for the combination of liposomal ADR (DOXIL®) with liposomal IL-2. In terms of number of surviving subjects, the effect of the combination treatment was greater than a combination of the effects of the individual treatments.

B2. Metastatic Lung Adenocarcinoma Model: IL-2 in MLV (Formulation B) and PEG-Derivatized SUV (SSL). In this experiment, BALB/c mice were injected intravenously with 5×10^5 M109 tumor cells (day 0). Free adriamycin or DOXIL®, respectively, were administered intravenously on day 7 (8 mg/kg), followed 3 days later by intravenous cytokine treatment. Liposomal IL-2 (Formulation A; PEGylated SUV containing IL-2) was given once daily (50,000 CU/mouse) on days 11, 14 and 17. Control groups received no treatment or received the IL-2 treatment alone.

Each group, consisting of 8-9 mice, was inspected for survival up to 100 days after tumor

inoculation. Results are shown in Table II and Figure 2.

TABLE II

GROUP	TREATMENT	NUMBER OF SURVIVING MICE/TOTAL	MEDIAN SURVIVAL (DAYS)
1	Control	0/8	43
2	ADR	2/8	56
3	DOXIL®	1/8	66
4	SSL-IL-2	0/8	41
5	DOXIL® + SSL-IL-2	7/9	> 100

As a comparison of groups 3-5 shows, the combined treatment with DOXIL® and liposomal IL-2 was significantly more effective than treatment with either liposomal component alone, particularly in terms of the number of subjects surviving for the duration of the test, *i.e.* 100 days or more (7 out of 9 compared to 0-1 out of 8). In this aspect, the combined treatment was significantly more effective than a combination of the effects derived from the individual therapies.

In a second, more extensive study, nine groups of BALB/c mice were injected intraperitoneally with 5×10^5 M109 tumor cells. Free adriamycin or DOXIL® (8 mg/kg) were administered intraperitoneally 7 days later, followed 3 days later by intravenous cytokine treatment. The cytokine, given once daily (50,000 CU/mouse) on days 10, 13 and 16, consisted of free IL-2, IL-2 in Formulation A (Stealth® PEGylated SUV), or IL-2 in Formulation B (9:1 molar DMPC/DMPG MLV).

Each group, consisting of 8 mice, and an untreated control group of 11 mice, were inspected for survival up to 120 days after tumor inoculation. Results are shown in Table III.

TABLE III

GROUP	TREATMENT	NUMBER OF TUMOR FREE MICE/TOTAL	MEDIAN SURVIVAL (DAYS)
1	Control	3/11	69
2	ADR	4/8	100
3	ADR/free IL-2	3/8	53
4	ADR/MLV-IL-2	4/8	92
5	ADR/SSL-IL-2	3/8	72
6	DOXIL®	4/8	102
7	DOXIL®/free IL-2	5/8	> 120
8	DOXIL®/MLV-IL-2	7/8	> 120
9	DOXIL®/SSL-IL-2	5/8	> 120

In this study, administration of free ADR and IL-2 showed little or no benefit over free ADR alone (groups 2-5). However, combinations of either free or liposomal IL-2 with the chemotherapeutic drug in liposomes (DOXIL®) showed clear benefits over administration of the drug alone (groups 6-9). Overall, the groups (8 and 9) treated with a combination of both components in liposomes showed superior results. Group 8, in particular, showed a high survival rate and almost a complete absence of tumors.

B3. Subcutaneous colon carcinoma model: IL-2 in MLV. In this test, 7 groups of BALB/c mice were injected in the footpad with 10^5 C26 colon carcinoma cells. Seven days later, 8 mg/kg free or liposomal adriamycin was administered i.v. Free or liposomal IL-2, as shown in Table IV, was administered i.p. according to the schedule described above. Results are shown in Table IV.

TABLE IV

GROUP	TREATMENT	NUMBER OF TUMOR FREE MICE, DAY 30	NUMBER OF TUMOR FREE MICE, DAY 65
1	Control	0/7	0/7
2	ADR	0/7	0/7
3	ADR/free IL-2	0/7	0/7
4	ADR/MLV-IL-2	1/8	0/8
5	DOXIL®	4/8	102
6	DOXIL®/free IL-2	0/7	2/8
7	DOXIL®/MLV-IL-2	6/8	4/8

As the data shows, administration of liposomal drug alone was somewhat beneficial, but only the group receiving the combined liposomal treatment showed significant recovery from tumors. In this group (group 7), it was also observed that the tumors were significantly smaller than in the other groups.

IV. Administration

For use in humans, a therapeutically effective dose of the composition typically corresponds to 20-100 mg adriamycin/m^2 of body surface. For IL-2, a preferred dose corresponds to 50,000 - 500,000 CU per square meter of body surface. Administration may be by intraperitoneal (ip), subcutaneous (sc), intravenous (iv), intraarterial (ia), or intramuscular (im) injection. Liposomes in the form of large multilamellar vesicles (MLV's) are preferred for intraperitoneal, subcutaneous or intramuscular administration, while SUV's are preferred for intravenous as well as intramuscular administration.

As shown above, administration of liposome-encapsulated chemotherapeutic drug is followed by administration of the liposome-encapsulated cytokine. While specific time intervals and courses of treatment have been shown in the examples above, it is understood that dosages, time intervals between courses, and the number of courses of treatment, for both drug and cytokine, may be varied depending on the extent of symptoms and the condition of the patient.

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications may be made without departing from the invention.

IT IS CLAIMED:

1. A method of antitumor therapy, comprising administering to a subject in need of such treatment, a therapeutically effect amount of a combination of components encapsulated in liposomes, wherein said components comprise a chemotherapeutic drug and an immunostimulating cytokine.

2. The method of claim 1, wherein said administering produces a greater therapeutic effect than a combination of the effects produced by the liposome-encapsulated components administered individually.

3. The method of claim 1, wherein the liposomes contain about 1-10 mole percent of a lipid having a polar head group derivatized with a polyethylene glycol (PEG) chain which has a molecular weight of between 750 and 10,000 daltons.

4. The method of claim 1, wherein the liposomes contain at least one lipid selected from the group consisting of dimyristoyl phosphatidyl choline, dimyristoyl phosphatidyl glycerol, 1,2-distearoyl-3-trimethylammonium propane, phosphatidyl choline, phosphatidyl ethanolamine, and cholesterol.

5. The method of claim 1, wherein the chemotherapeutic drug is encapsulated in vesicles having a mean diameter of approximately 50 to 120 nm, and containing about 1-10 mole percent of a lipid having a polar head group derivatized with a polyethylene glycol (PEG) chain which has a molecular weight of between 750 and 10,000 daltons.

6. The method of claim 1, wherein the chemotherapeutic drug is selected from a chemotherapeutic anthraquinone, cis-platin, and a topoisomerase I inhibitor.

7. The method of claim 6, wherein the chemotherapeutic anthraquinone is adriamycin.

8. The method of claim 6, wherein the topoisomerase I inhibitor is camptothecin or a camptothecin analog.

9. The method of claim 7, wherein the chemotherapeutic drug encapsulated in liposomes is DOXIL®.

10. The method of claim 1, wherein the cytokine is selected from the group consisting of

interleukin-2 (IL-2), IL-12, IL-15, IL-18, IFN-γ, IFN-α, IFN-β, TNF-α, G-CSF, and GM-CSF.

11. The method of claim 10, wherein the cytokine is IL-2.

12. The method of claim 1, wherein the cytokine is encapsulated in liposomes comprising dimyristoyl phosphatidyl choline plus 0 - 50 mole percent of at least one lipid selected from dimyristoyl phosphatidyl glycerol and 1,2-distearoyl-3-trimethylammonium propane.

13. The method of claim 1, wherein administration of the liposome-encapsulated cytokine follows administration of the liposome-encapsulated chemotherapeutic drug.

14. A method of antitumor therapy, comprising administering to a subject in need of such treatment, a therapeutically effect amount of a combination of a chemotherapeutic drug and an immunostimulating cytokine, wherein the chemotherapeutic drug is encapsulated in liposomes which contain about 1-10 mole percent of a lipid having a polar head group derivatized with a poly-ethylene glycol (PEG) chain which has a molecular weight of between 750 and 10,000 daltons.

15. The method of claim 14, wherein said administering produces a greater therapeutic effect than a combination of the effects produced by the liposome-encapsulated drug and the cytokine administered individually.

16. The method of claim 14, wherein the chemotherapeutic drug is selected from a chemotherapeutic anthraquinone, cis-platin, and a topoisomerase I inhibitor.

17. The method of claim 16, wherein the chemotherapeutic anthraquinone is adriamycin.

18. The method of claim 17, wherein the chemotherapeutic drug encapsulated in liposomes is DOXIL®.

19. The method of claim 14, wherein the topoisomerase I inhibitor is camptothecin or a camptothecin analog.

20. The method of claim 14, wherein the cytokine is selected from the group consisting of interleukin-2 (IL-2), IL-12, IL-15, IL-18, IFN-γ, IFN-α, IFN-β, TNF-α, G-CSF, and GM-CSF.

21. The method of claim 20, wherein the cytokine is IL-2.

22. The method of claim 14, wherein administration of the cytokine follows administration of

the liposome-encapsulated chemotherapeutic drug.

23. A composition for use in antitumor therapy, comprising a combination of components encapsulated in liposomes, wherein said components comprise a chemotherapeutic drug and an immunostimulating cytokine.

24. The composition of claim 23, wherein said combination is effective to produce, in a tumor-afflicted subject, a greater antitumor effect than a combination of the effects produced by the liposome-encapsulated components administered individually.

25. The composition of claim 23, wherein the chemotherapeutic drug is encapsulated in liposomes containing about 1-10 mole percent of a lipid having a polar head group derivatized with a polyethylene glycol (PEG) chain which has a molecular weight of between 750 and 10,000 daltons.

26. The composition of claim 23, wherein the chemotherapeutic drug is selected from a chemotherapeutic anthraquinone, cis-platin, and a topoisomerase I inhibitor.

27. The composition of claim 26, wherein the chemotherapeutic anthraquinone is adriamycin.

28. The composition of claim 27, wherein the chemotherapeutic drug encapsulated in liposomes is DOXIL®.

29. The composition of claim 23, wherein the cytokine is selected from the group consisting of interleukin-2 (IL-2), IL-12, IL-15, IL-18, IFN-γ, IFN-α, IFN-β, TNF-α, G-CSF, and GM-CSF.

30. The composition of claim 29, wherein the cytokine is IL-2.

1 / 2

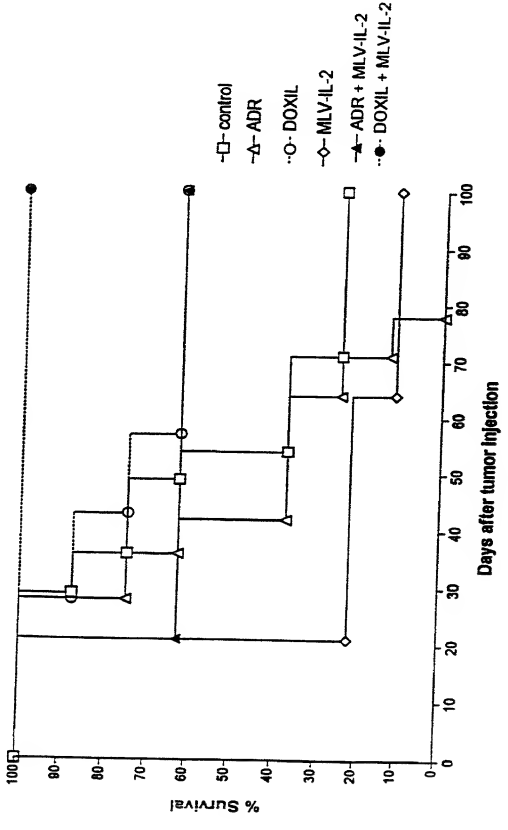

Fig. 1

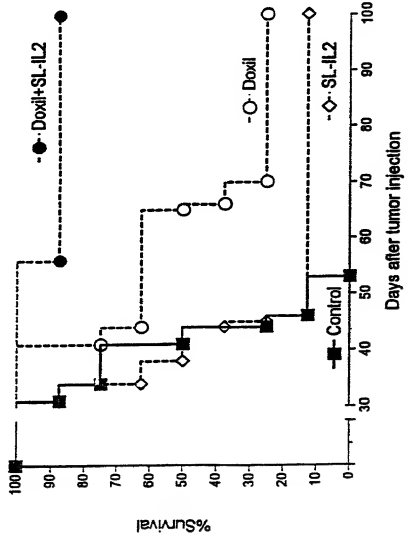

Fig. 2

COMBINED DECLARATION AND POWER OF ATTORNEY
ncludes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER
9325-0007.10

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

COMBINED CHEMO-IMMUNOTHERAPY WITH LIPOSOMAL DRUGS AND CYTOKINES

the specification of which (check only one item below):

☐ is attached hereto.

☐ was filed as United States application Serial No. _____ on _____.
and was amended on _____ (if applicable)

☒ **was filed as PCT international application Number PCT/IL98/00586 on 01 December 1998.**
and was amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56(a).

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate or 365(a) of any PCT international application(s) which designated at least one country other than the United States of America listed below and have also identified below, by checking the box, any foreign application(s) for patent or inventor's certificate, or of any PCT international application(s) having a filing date before that of the application(s) on which priority is claimed.

RIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. §119:

COUNTRY (if PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC §119	
			☐YES	☐ NO

☐ **I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or 365(c) of any PCT international application(s) designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT application(s) in the manner provided by the first paragraph of 35, U.S.C. §112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56(a) which became available between the filing date of the prior application(s) and the national or PCT international filing ate of this application.**

RIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. §120:

U.S. APPLICATIONS			STATUS (Check one)		
U.S. APPLICATION NUMBER	U.S. FILING DATE		PATENTED	PENDING	ABANDONED
60/067,697	**4 December 1997**				**X**
PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO.	PCT FILING DATE	U.S. SERIAL Nos.ASSIGNED (if any)			
PCT/IL98/00586	**01 December 1998**			**X**	

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to rosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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Linda R. Judge 42,702 Michael L. Gencarella 44,703 Michael T. Gabrik 32,896
Gregory L. Heinkel 44,755

OMBINED DECLARATION AND POWER OF ATTORNEY (CONTINUED)
ncludes Reference to PCT International Applications)

ATTORNEY'S DOCKET NO.
9325-0007.10

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reby declare that all statements made herein of my own knowledge are true and that alls statements made on information and belief are believed to be true, and further that these statements were made with th knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements ma peardize the validity of the application or any patent issuing thereon

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
DATE June 4, 2000	DATE June 4, 2000	DATE June. 4. 2000

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C., 20231, on:

Date: 7.31.00 By: [signature]

Docket No. 9325-0007.10 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF:
Alberto A. Gabizon, *et al.*
SERIAL NO.: not yet assigned
FILED: June 2, 2000
FOR: **COMBINED CHEMO-IMMUNOTHERAPY WITH LIPOSOMAL DRUGS AND CYTOKINES**

EXAMINER: Unknown
ART UNIT: Unknown

Power of Attorney by Assignee and Certification Under 37 CFR §3.73(b)

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, the undersigned, acting on behalf of the Assignee of

☒ an undivided share of the entire right, title, and interest

☐ the entire right, title and interest

in the above-identified patent application, appoint the attorneys and agents listed below to prosecute this application and transact all business with the U.S. Patent and Trademark Office in connection therewith. This appointment is to the exclusion of the inventor(s) and their attorney(s) and agent(s) in accordance with the provisions of 37 CFR 3.71.

All prior powers of attorney for this application are hereby revoked. The appointed representatives are:

7 —

Peter J. Dehlinger, Registration No. 28,006
Judy M. Mohr, Registration No. 38,563
LeeAnn Gorthey, Registration No. 37,337
Linda R. Judge, Registration No. 42,702
Michael L. Gencarella, Registration No. 44,703
Michael T. Gabrik, Registration No. 32,896
Gregory L. Heinkel, Registration No. 44,755

all affiliated with Iota Pi Law Group.

Direct all telephone calls to LeeAnn Gorthey at (650) 324-0880. Address all correspondence to:

Iota Pi Law Group
P.O. Box 60850
Palo Alto, CA 94306
Telephone: (650) 324-0880

In accordance with 37 CFR 3.73(b), I hereby certify that I am empowered to act on behalf of the Assignee. To the best of my knowledge and belief, title is in the Assignee, as evidenced by the Assignment noted above.

I further declare that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, USC §1001 and that such willful false statements may jeopardize the validity of the this application or any patent resulting therefrom.

ASSIGNEE: Hadasit Medical Research Services and Development, Ltd.

Signature: _____

Typed Name: _____

Title: _____

Date: _____

Address: _____

הדסית
שרותי מחקר רפואי ופתוח בע"מ
hadasit
medical research services
& development ltd.

RAPHAEL HOFSTEIN PH.D
MANAGING DIRECTOR
HADASIT MEDICAL RESEARCH
SERVICE & DEVELOPMENT LTD

19/6/2000

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C., 20231, on:

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Assistant Commissioner for Patents
Washington, D.C. 20231

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☒ an undivided share of the entire right, title, and interest

☐ the entire right, title and interest

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I further declare that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, USC §1001 and that such willful false statements may jeopardize the validity of the this application or any patent resulting therefrom.

ASSIGNEE: Yissum Research Development Company of the Hebrew University of Jerusalem

Signature: _____

Typed Name: Mordehai Perlmutter

Title: Managing Director and CEO

Date: June 19, 2000

Address: 46 Jabotinsky St, P.O.Box 4279, Jerusalem 91042, Israel